Appendix C

Final Background Review Document:

The Nonradioactive Murine Local Lymph Node Assay: DA

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Background Review Document
Nonradioactive Murine Local Lymph Node Assay: DA

Interagency Coordinating Committee on the
Validation of Alternative Methods

National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

March 2010

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
Anim.	Animal
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
aq.	Aqueous
BRD	Background review document
Calc.	Calculated
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
CI	Confidence interval
Conc.	Concentration
CV	Coefficient of variation
Cys	Cysteine-containing peptide
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2	Estimated concentration needed to produce a stimulation index of two
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index of a specified threshold
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
FN	False negative
FP	False positive
GP	Guinea pig
GPMT	Guinea pig maximization test
HMT	Human maximization test
HPTA	Human patch test antigen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IDR	Insufficient dose response
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods

K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LLNA: BrdU-ELISA	Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
MEK	Methyl ethyl ketone
MHLW	Ministry of Health, Labour and Welfare (Japan)
Min	Minimal
Mod	Moderate
Mol.	Molecular
NA	Not applicable
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NR	Not reported
NT	Not tested
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PC	Positive control
Ref.	Reference
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SD	Standard deviation
SI	Stimulation index
SLS	Sodium lauryl sulfate
Stats.	Statistics
TG	Test guideline
Trad.	Traditional
U.S.	United States
Unk	Unknown
VC	Vehicle control
Veh.	Vehicle
vs.	Versus

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Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for allergic contact dermatitis testing. It is now commonly used around the world.

One disadvantage of the traditional LLNA is that it requires injection of a radioactive marker to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers, scientists have recently developed several nonradioactive versions of the LLNA. In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate the scientific validity of these nonradioactive versions. ICCVAM assigned the nomination a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the validity of three such test methods. The evaluation process involved two public meetings of an international independent scientific peer review panel (referred to hereafter as "Panel") that reviewed draft and revised draft background review documents and ICCVAM test method recommendations.

A comprehensive draft background review document (BRD) provided the initial information, data, and analyses supporting the validation status of each of the nonradioactive test methods. ICCVAM also developed draft test method recommendations for each test method regarding its usefulness and limitations, test method protocol, performance standards, and future studies. NICEATM and ICCVAM provided the draft BRDs and draft test method recommendations to the Panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[1] Both the Panel and ICCVAM concluded that more information was needed before a recommendation on the usefulness and limitations of each of the three test methods could be made. The Panel recommended that NICEATM obtain additional existing data that were not available to the Panel and reanalyze the performance of each nonradioactive LLNA test method. NICEATM subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised draft BRDs. NICEATM and ICCVAM provided the revised draft BRDs and revised draft test method recommendations to the Panel for their consideration at a public meeting on April 28-29, 2009. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[2]

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") that was circulated in July 2009 to the 30 OECD member countries for review and comment. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to the comments from member countries. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment and then the final draft was

[1] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf.
[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf.

forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM considered the conclusions and recommendations of the Panel and conclusions from the OECD Expert Consultation, along with comments received from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (the ICCVAM-NICEATM advisory committee), and then finalized the BRDs and test method recommendations. These will be forwarded to Federal agencies for their consideration and acceptance decisions, where appropriate. This BRD addresses the validation database for the LLNA: DA.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Kim Headrick, and Dr. Stephen Ullrich for their service as Evaluation Group Chairs. We thank Drs. Abigail Jacobs (U.S. Food and Drug Administration) and Joanna Matheson (CPSC) for serving as Co-chairs of the IWG, as well as the members of the IWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final BRD.

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Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an international independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[3] The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Organization for Standardization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM.[4] One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of data and information regarding the usefulness and limitations of one of these test methods, the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA").

Test Method Protocol

Daicel Chemical Industries, Ltd. developed the LLNA: DA test method based on modifications to the traditional LLNA (Yamashita et al. 2005). While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactivity into the DNA of dividing lymph node cells, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the timing and administration of the test substance. In the traditional LLNA, the test substance is applied on days 1, 2, and 3 and the auricular lymph nodes are excised on day 6. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7 and the auricular lymph nodes are excised on day 8. Furthermore, one hour prior to each application of the test substance, 1% aqueous solution of sodium lauryl sulfate is applied to increase absorption of the test substance through the skin. A stimulation index (SI) is used to identify a substance as a sensitizer (the ratio of the mean ATP content of the substance treatment group to the mean ATP content of the vehicle treatment group).

Validation Database

The accuracy and reliability of the LLNA: DA were assessed using data submitted to NICEATM for 45 substances tested in one laboratory (Idehara et al. 2008; Idehara unpublished) and 14 substances

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[3] Hhttp://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdfH.

[4] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdfH.

tested in a two-phased interlaboratory validation study (17 laboratories) (Omori et al. 2008). Of the 14 substances tested in the two-phased interlaboratory study (Omori et al. 2008) only one was different from the 45 substances tested initially (Idehara et al. 2008; Idehara unpublished). Thus, data were available for 46 unique substances tested in the LLNA: DA. The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests. One substance, benzocaine, yielded both positive and negative results in the traditional LLNA (ICCVAM 1999) and therefore was not considered in the performance evaluation of the LLNA: DA. LLNA studies for another substance, toluene 2,4-diisocyanate (van Och et al. 2000), were not conducted according to the traditional LLNA test method protocol described (ICCVAM 1999; Dean et al. 2001). Thus of the 46 substances with LLNA: DA data, 44 substances had adequate traditional LLNA data (32 were classified by the traditional LLNA as skin sensitizers and 12 were classified as nonsensitizers).

Test Method Accuracy

The accuracy evaluation in this BRD includes the evaluation of multiple decision criteria, including the SI ≥ 3.0 recommended by the test method developer. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI ≥ 1.8 to classify potential skin sensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). The three false positive substances produced SI values between 1.8 and 2.5 in the LLNA: DA.

When the decision criterion of SI ≥ 3.0 was used to classify sensitizers versus nonsensitizers, compared to the traditional LLNA, accuracy was 91% (40/44), with a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32). Among the four discordant substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

The reduced LLNA: DA (rLLNA: DA), which uses only the highest dose of the test substance that does not elicit excessive skin irritation and/or systemic toxicity, has the potential to reduce animal use by up to 40% for hazard classification purposes when dose-response information is not needed. Using SI ≥ 1.8 to classify potential sensitizers for 123 individual tests which used multiple doses, overall accuracy of the rLLNA: DA compared to the multi-dose LLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the LLNA: DA at a concentration lower than the highest dose (maximum SI = 1.97 and 2.00). The highest dose tested for each of the two tests of the two substances was 50%.

Test Method Reliability – Intralaboratory Reproducibility

Intralaboratory reproducibility for the LLNA: DA was assessed using data for two substances (isoeugenol and eugenol) that were tested at varying concentrations in three different experiments. The coefficient of variation (CV) for the reproducibility of the EC3 values (estimated concentration needed to produce an SI of three) for isoeugenol and eugenol was 21% and 11%, respectively. The CV for the reproducibility of the EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol was 36% and 23%, respectively.

Test Method Reliability – Interlaboratory Reproducibility

This BRD includes a reproducibility analysis using SI ≥ 1.8 to identify potential sensitizers. The two-phased multilaboratory validation study included 17 different laboratories in which 14 different substances were examined. In the first phase of the study, 10 laboratories each tested up to 12 substances, while in the second phase of the study seven laboratories (different from the 10 laboratories in the first phase of the interlaboratory validation study) each tested up to five substances (2/5 substances unique compared to the first phase). In both studies, each substance was tested once at

three different doses, which were provided to the participating laboratories by the validation study management team.

When using SI ≥ 1.8 as the decision criterion, the qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested in up to 10 laboratories during the first phase of the LLNA: DA interlaboratory validation study resulted in 100% (3/3 or 10/10) concordance for 9 substances (seven sensitizers and two nonsensitizers in the traditional LLNA), 90% (9/10) concordance for one substance (one nonsensitizer in the traditional LLNA), and 67% (2/3) concordance for two substances (two sensitizers in the traditional LLNA). The coefficient of variation (CV) values for the estimated concentration needed to produce a stimulation index of 1.8 (EC1.8) values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71%. The qualitative interlaboratory concordance analysis for the five substances tested in up to seven laboratories during the second phase of the validation study resulted in 100% (4/4 or 7/7) concordance for four substances (three sensitizers and one nonsensitizer in the traditional LLNA) and 75% (3/4) concordance for one substance (a sensitizer in the traditional LLNA). The CV values for the EC1.8 values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49%.

When using SI ≥ 1.8 to classify potential sensitizers, the tally of concordant tests for the 14 substances with multiple LLNA: DA tests indicated that the SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8). The concordance of the other two sensitizers (based on traditional LLNA results) was 50% (4/8) to 67% (2/3) for SI ≥ 1.8. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≤ 1.8). The concordance of the other nonsensitizer (based on traditional LLNA results) was 91% (10/11) for SI ≤ 1.8.

Animal Welfare Considerations

The LLNA: DA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (ICCVAM 2009). However, since use of the traditional LLNA is restricted in some institutions because it involves radioactivity, availability and use of the nonradioactive LLNA: DA may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure.

Further, the LLNA: DA evaluates the induction phase of sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: DA protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the guinea pig maximization test [GPMT]).

Test Method Transferability

The transferability of the LLNA: DA was demonstrated by a two-phased interlaboratory validation study (Omori et al. 2008). Notably, the test method developer indicates that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). Compared to the traditional LLNA, the LLNA: DA will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA except that the understanding and practice of luciferase methodology is required.

1.0 Introduction

1.1 Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem that often results in lost workdays[5] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing through the epidermis, where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

The elicitation phase occurs when the individual is again topically exposed to the same substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and presented to circulating T-lymphocytes. The antigen-specific T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Sailstad et al. 2001; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[6] ICCVAM forwarded recommendations to U.S. Federal agencies that the LLNA should be considered for regulatory acceptance or other nonregulatory applications for assessing the ACD potential of substances, while recognizing that some testing situations would still require the use of traditional GP test methods (ICCVAM 1999; Sailstad et al. 2001). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline [TG] 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM.[7] One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. This BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these test methods, the LLNA modified by Daicel Chemical

[5] http://www.bls.gov/IIF

[6] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.

[7] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdfH.

Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") in the draining auricular lymph nodes. ICCVAM and its Immunotoxicity Working Group (IWG) evaluated this method in a draft BRD and developed draft test method recommendations based on this initial evaluation.

A Panel reviewed the draft BRD in March 2008 to evaluate the extent to which the information contained in the draft BRD supported the draft test method recommendations. The Panel concluded that additional information was needed to evaluate the test method, including a detailed test method protocol, quantitative data for the test method, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM obtained additional LLNA: DA data and information, which were used to generate a revised draft BRD for review by the Panel in April 2009.

Based on the revised draft ICCVAM test method recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences, the Environmental Protection Agency, the Food and Drug Administration, and CPSC, as well as U.S. and international experts from industry and other stakeholder organizations, participated in this meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was distributed to the 30 OECD member countries in December 2009, via their National Co-ordinators, for review and comment by national experts and interested stakeholders. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010 to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM and the IWG considered the conclusions and recommendations of the Panel, comments received from the public and its advisory committee (the Scientific Advisory Committee on Alternative Toxicological Methods), along with the conclusions of the OECD Expert Consultation on the LLNA, and developed this final BRD. ICCVAM provides this final BRD to regulatory agencies for consideration as part of the ICCVAM Test Method Evaluation Report.

1.3 The LLNA: DA

Daicel Chemical Industries, Ltd. developed the LLNA: DA as a nonradioactive modification (Yamashita et al. 2005; Idehara et al. 2008) to the traditional LLNA. The traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing lymph node cells. In contrast, the LLNA: DA assesses increases in ATP content in the draining auricular lymph nodes by employing a luciferin-luciferase assay to measure bioluminescence. Since ATP content is linearly related to living cell number, this measurement serves as a surrogate for cell number at the time of sampling (Crouch et al. 1993).

This document provides:

- A comprehensive summary of the LLNA: DA test method protocol
- The substances used in the validation of the test method and the test results
- The performance characteristics (accuracy and reliability) of the test method
- Animal welfare considerations
- Other considerations relevant to the usefulness and limitations of this test method (e.g., transferability, cost of the test method)

2.0 LLNA: DA Test Method Protocol

This BRD includes the detailed standard operating procedure for the LLNA: DA test method that was used in the validation studies (**Annex I**). The LLNA: DA test method protocol (**Annex I**) differs from the ICCVAM-recommended test method protocol for the traditional LLNA (ICCVAM 2009) in the method used to assess lymphocyte proliferation in the auricular lymph nodes (**Table C-1**). In addition, there are substantive differences between the two test method protocols regarding test substance application and timing for the collection of the lymph nodes. In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, radiolabeled thymidine or iodine is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and radioactive thymidine or iodine incorporation is determined by β-scintillation or γ-scintillation counting, respectively. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and additionally on day 7. During the initial development of the LLNA: DA, the study group (Yamashita et al. 2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth application (day 7) was useful for increasing lymph node proliferation. Based on a statistically significant increase in lymph node weight-based stimulation index (SI) values for mice that received a fourth application (day 7) of the test substance, this test method protocol was chosen. Furthermore, one hour prior to each application of the test substance, an aqueous solution of 1% sodium lauryl sulfate (SLS) is applied to the dorsum of the treated ears to increase absorption of the test substance across the skin (van Och et al. 2000). Various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002). Idehara et al. (2008) observed similar results (see also **Annex I** for supplemental data submitted to NICEATM evaluating the effect of 1% SLS pretreatment on lymph node cell proliferation [Idehara unpublished]). Lastly, 24 to 30 hours after the last test substance application on day 7, the auricular lymph nodes are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay (day 8). The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000). It utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin according to the following reaction:

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer.

Table C-1 Comparison of the LLNA: DA and Traditional LLNA Experimental Procedure

Day	LLNA: DA	Traditional LLNA
1, 2, & 3	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	• Apply 25 µL of test substance or vehicle to dorsum of each ear
4 & 5	• No treatment	• No treatment
6	• No treatment	• Administer ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells
7	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	• No treatment
8	• Excision of auricular lymph nodes • Measurement of ATP content in lymph node cells	• No treatment

Abbreviations: ^{3}H = tritiated; ^{125}I = iodine-125; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SLS = sodium lauryl sulfate.

2.1 Decision Criteria

Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin sensitizers from nonsensitizers. The formula for calculating the SI in the LLNA: DA is the ratio of the mean ATP content of the auricular lymph nodes collected from the test substance treatment group to the mean ATP content of the auricular lymph nodes collected from the vehicle treatment group (measured in relative luminescence units; RLU):

$$SI = \frac{\text{mean ATP content of auricular lymph nodes in test treatment group (RLU)}}{\text{mean ATP content of auricular lymph nodes in vehicle treatment group (RLU)}}$$

In the intra- and interlaboratory validation studies for the LLNA: DA, an SI ≥ 3.0 was used as the threshold for identifying a substance as a sensitizer, which is the same threshold used in the traditional LLNA. As noted in **Section 6.0**, alternative decision criteria are evaluated in this BRD to determine the threshold that provides optimum performance.

3.0 LLNA: DA Validation Database

To evaluate the usefulness and limitations of the LLNA: DA, Daicel Chemical Industries, Ltd. tested a total of 45 substances in one laboratory (Idehara et al. 2008; Idehara unpublished). They further evaluated two of the 45 substances (isoeugenol and eugenol) in the LLNA: DA at varying concentrations in three different experiments in order to assess intralaboratory reproducibility. In addition, a two-phased interlaboratory validation study evaluated the reproducibility of the LLNA: DA (**Section 7.0**). In the first phase 10 laboratories tested 12 coded substances and in the second phase seven different laboratories tested five coded substances. Between the 17 laboratories, 14 different substances were examined and one of those substances, 3-aminophenol, was not previously tested among the 45 substances in the intralaboratory validation study, yielding a total of 46 substances tested in the LLNA: DA.

All 46 substances tested in the LLNA: DA were previously tested in the traditional LLNA, including 40 substances that were considered in the original ICCVAM evaluation of the traditional LLNA (ICCVAM 1999). Cinnamic alcohol, diethyl maleate, ethyl acrylate, glutaraldehyde, methyl methacrylate, and toluene 2,4-diisocyanate were the six substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report.

Of the 46 substances tested in the LLNA: DA, 33 were classified by the LLNA as skin sensitizers,[8] 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results and therefore was not included in the performance analyses (ICCVAM 1999)[9] (**Table C-2**). For the sensitizers in the LLNA, the range of traditional LLNA EC3 values (estimated concentrations needed to produce an SI of three) was from 0.009% to 90% (**Table C-2**). Similar to benzocaine, LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM 1999 report, were not suitable for comparison. The LLNA test method protocol followed for the study that tested toluene 2,4-diisocyanate (van Och et al. 2000) was a modified version of the traditional LLNA which was not performed in accordance with OECD TG 429 (OECD 2002) or ICCVAM 1999 and Dean et al. (2001). One variation included use of the BALB/c strain of mouse for the experiments, and not the CBA/Ca or CBA/J strains as specified by ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the mice were pretreated with an aqueous solution of 1% SLS before treatment with the test substance. The authors also stated that the auricular lymph nodes were excised and pooled for each animal. Thus, of the 46 substances with LLNA: DA and LLNA data, 44 had adequate traditional LLNA data and were included in the accuracy analyses described in **Section 6.0**.

Annex II provides information on physicochemical properties (e.g., physical form tested). For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

Annex II further provides information on the Chemical Abstracts Service Registry Number (CASRN) and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine Medical Subject Headings. If

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[8] Resorcinol was classified as a nonsensitizer based on original LLNA data (ICCVAM 1999) but recent LLNA data have instead suggested that it is actually a sensitizer (Basketter et al. 2007a) and is therefore classified as a sensitizer for this evaluation.

[9] A series of 12 tests conducted in two laboratories resulted in some positive results that were not reproducible (Basketter et al. 1995).

chemical classes were not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system.[10] A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential. Instead, chemical class information is being presented to provide an indication of the variety of structural elements that are present in the substances that were evaluated in this analysis.

Table C-2 shows that 20 chemical classes are represented by the 44 substances tested in the LLNA: DA with adequate traditional LLNA data; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (five substances). Further, of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates, [Gerberick et al. 2004]), only ketones were not included in the LLNA: DA database.

[10] Hhttp://www.nlm.nih.gov/mesh/meshhome.htmlH.

Table C-2 Product Use, Chemical Classification, and Traditional LLNA EC3 Values of 46 Substances Tested in the LLNA: DA

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one[5]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	1
p-Benzoquinone[5]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	1
2,4-Dinitrochlorobenzene[6, 7]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	15
Benzalkonium chloride[6]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[8] (11.1)	1
Glutaraldehyde[6, 7]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	3
p-Phenylenediamine[6]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	6
Toluene 2,4-diisocyanate[6, 9]	Intermediate in chemical synthesis	Hydrocarbons, Cyclic; Isocyanates	0.110 (NR)	1
Potassium dichromate[6, 10]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	12
Propyl gallate[5]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	1
Phthalic anhydride[6]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	1
Formaldehyde[6, 7]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	4
Cobalt chloride[6, 7, 10]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	2

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol[6, 7]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	47
2-Mercaptobenzothiazole[6]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	1
Cinnamic aldehyde[6]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	6
3-Aminophenol[7]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1
Benzocaine[6]	Medication	Carboxylic Acids	3.400[11] (7.6)	1
Diethyl maleate[5]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	4
Trimellitic anhydride[6]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	2
Nickel (II) sulfate hexahydrate[6, 7, 10]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	1
Resorcinol[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	1
Sodium lauryl sulfate[6]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	5
Citral[6]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	6
Hexyl cinnamic aldehyde[6, 7, 10]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	21

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Eugenol[6]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	11
Abietic acid[6, 7]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	5
Phenyl benzoate[5]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	3
Cinnamic alcohol[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	1
Hydroxycitronellal[6]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	6
Imidazolidinyl urea[6]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	1
Ethylene glycol dimethacrylate[5]	Manufacturing	Carboxylic Acids	28.000 (7.0)	1
Butyl glycidyl ether[5]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	1
Ethyl acrylate[5]	Manufacturing	Carboxylic Acids	32.800 (4.0)	2
Methyl methacrylate[5]	Manufacturing	Carboxylic Acids	90.000 (3.6)	1
1-Bromobutane[6]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	1
Chlorobenzene[6]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	1

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Diethyl phthalate[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	1
Dimethyl isophthalate[5, 7]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	1
Hexane[6]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	1
Isopropanol[6, 7]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	1
Lactic acid[6, 10]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	1
Methyl salicylate[6, 7]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	9
Propylparaben[6]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	1
Nickel (II) chloride[5]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	2
Salicylic acid[5]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	1
Sulfanilamide[5]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	1

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not applicable; NR = not reported; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database (HSDB)-National Library of Medicine-TOXNET .http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program .http://hazmap.nlm.nih.gov/.
Household Products Database-National Library of Medicine .http://hpd.nlm.nih.gov/index.htm
International Programme on Chemical Safety (IPCS) INCHEM database in partnership with Canadian Centre for Occupational Health and Safety (CCOHS) .http://www.inchem.org/.
National Toxicology Program .http://ntp.niehs.nih.gov:8080/index.html?col=010stat

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: .http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 value (estimated concentration needed to produce a stimulation index of three) listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA (**Annex IV)**, except where noted. Numbers in parentheses indicate the maximum stimulation index, where reported.

[4] Number of traditional LLNA studies from which the data were obtained.

[5] Substance tested in intralaboratory validation study (Idehara unpublished).

[6] Substance tested in intralaboratory validation study (Idehara et al. 2008).

[7] Substance tested in first phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[8] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle (**Annex IV**) but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[9] Not included in accuracy analyses. Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

[10] Substance tested in second phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[11] Not included in accuracy analyses. EC3 value reported in **Table C-2** for benzocaine is based on data from the NICEATM database but variable and equivocal (i.e., results that were not reproducible) responses were reported by Basketter et al. (1995) and in the 1999 ICCVAM report.

4.0 Reference Data

As mentioned in **Section 3.0**, 44 of the 46 substances tested in the LLNA: DA have adequate traditional LLNA data and are included in the accuracy analyses described in **Section 6.0.** The traditional LLNA reference data used for the accuracy analyses comparisons are from ICCVAM (1999) (**Annex III**) for 34 of those 44 substances. The traditional LLNA reference data for the remaining 10 substances (benzalkonium chloride, cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate, formaldehyde, glutaraldehyde, imidazolidinyl urea, methyl methacrylate, and nickel [II] sulfate hexahydrate) were obtained from other sources (**Annex III**) (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006). In addition, Basketter et al. (2007a) reassessed the skin sensitization potential of resorcinol in the LLNA, in accordance with OECD TG 429 (2002), which updates information in the ICCVAM 1999 report and from Gerberick et al. (2005) that had previously stated that this substance tested negative in the LLNA.

The reference data for the GP tests (guinea pig maximization test or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Vandenberg and Epstein (1963), Kligman (1966a, 1966b, 1966c), Marzulli and Maibach (1974), Jordan and King (1977), Klecak et al. (1977), Marzulli and Maibach (1980), Van der Walle et al. (1982), Gad et al. (1986), Robinson et al. (1990), Gerberick et al. (1992), ICCVAM (1999), Basketter et al. (1999a, 1999b, 2001, 2005, 2007a), Kwon et al. (2003), Schneider and Akkan (2004), and Betts et al. (2006).

An independent quality assurance contractor for the National Toxicology Program audited the traditional LLNA data provided in the ICCVAM 1999 report. Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences. The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records.

5.0 LLNA: DA Test Method Data and Results

The test method data in this BRD include the individual animal data for the LLNA: DA results from the validation studies by Idehara et al. (2008) and Omori et al. (2008). In addition, individual animal data for 14 unpublished studies (Idehara unpublished) were submitted to NICEATM and were included in the evaluation (although the individual animal data were submitted to NICEATM they are not included in the BRD at the request of the test method developer since they are not yet published). **Annex III** represents a summary of data for the 46 different substances tested in the LLNA: DA, and includes the comparative traditional LLNA data that were available for 44 of the 46 substances (see also **Section 3.0**). In addition, 42 of the 46 substances examined in the LLNA: DA have GP data and 43 of the 46 substances tested have human skin sensitization data. Based on Idehara et al. (2008; unpublished), the 45 substances tested in the intralaboratory study were not coded prior to testing. However, the two-phased interlaboratory validation study used coded substances (Omori et al. 2008). Original data for these studies are included in **Annex IV**.

6.0 LLNA: DA Test Method Accuracy

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed test method when compared to the current reference test method (ICCVAM 2003). Additional comparisons should also be made against any available human data or experience from testing or accidental exposures. This aspect of assay performance is typically evaluated by calculating:

- *Accuracy (concordance)*: the proportion of correct outcomes (positive and negative) of a test method
- *Sensitivity*: the proportion of all positive substances that are classified as positive
- *Specificity*: the proportion of all negative substances that are classified as negative
- *False positive rate*: the proportion of all negative substances that are incorrectly identified as positive
- *False negative rate*: the proportion of all positive substances that are incorrectly identified as negative

6.1 LLNA: DA Database Used for the Accuracy Analysis

An accuracy analysis for the LLNA: DA test method was conducted using data from the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory validation study (Omori et al. 2008). Taken together, LLNA: DA test data were available for 46 different substances, 44 of which had adequate comparative traditional LLNA data to conduct an accuracy analysis (**Section 3.0**). Thus, of the 44 substances included in the accuracy analysis, 40 had LLNA: DA, traditional LLNA, and GP data and 41 had LLNA: DA, traditional LLNA, and human data. Classification of substances and data available for each substance are provided in **Annex III**.

Multiple LLNA: DA tests were available for 14 substances tested in the intralaboratory (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory LLNA: DA studies (Omori et al. 2008). For the accuracy analyses, the test results were combined so that each substance was represented by one overall result for the SI analyzed and represented the outcome that was most prevalent. For example, when using SI $\geq$ 3.0 as the decision criterion, cobalt chloride was positive because five of the eight LLNA: DA results were positive (**Annex IV**). Also, using SI $\geq$ 3.0 as the decision criterion, inconsistent test results were noted for two of the 14 substances with multiple test results: cobalt chloride and nickel (II) sulfate hexahydrate. Three of the validation laboratories that tested cobalt chloride reported SI < 3.0 and five laboratories yielded SI $\geq$ 3.0. For nickel (II) sulfate hexahydrate, six validation laboratories reported SI < 3.0 and two laboratories yielded SI $\geq$ 3.0.

6.2 Accuracy Analysis Using the SI $\geq$ 3.0 Decision Criterion

The performance characteristics of the LLNA: DA test method were first evaluated using the decision criterion of SI $\geq$ 3.0 to identify sensitizers, which was the threshold for a positive response used in both the intralaboratory and two-phased interlaboratory validation studies (**Annex I**).

6.2.1 Accuracy vs. the Traditional LLNA

Based on the data (44 substances), when compared to the traditional LLNA, the LLNA: DA had an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-3**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared for substances with LLNA: DA, traditional LLNA, and GP data, and GP results served as the reference data, the LLNA: DA had a lower accuracy (78% [31/40] vs. 85% [34/40]), sensitivity (85% [22/26]

vs. 96% [25/26]), the same specificity (64% [9/14]) and false positive rate (36% [5/14]), and higher false negative rate (15% [4/26] vs. 4% [1/26]) relative to the traditional LLNA (**Table C-3**).

6.2.3 Accuracy vs. Human Data

When substances with only comparative LLNA: DA, traditional LLNA, and human data were evaluated, and human outcomes served as the reference point, the LLNA: DA had lower accuracy (76% [31/41] vs. 85% [35/41]) and sensitivity (74% [26/35] vs. 86% [30/35]), the same specificity (83% [5/6]) and false positive rate (17% [1/6]), and higher false negative rate (26% [9/35] vs. 14% [5/35]) relative to the traditional LLNA (**Table C-3**).

Table C-3 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Using Decision Criterion of SI ≥ 3.0 to Identify Sensitizers

Comparison	n[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32	100 (28/28)	75 (12/16)
Substances with LLNA: DA, Traditional LLNA, and GP Data								
LLNA: DA vs. Traditional LLNA	40	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
LLNA: DA vs. GP[3]	40	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
Traditional LLNA vs. GP[3]	40	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data								
LLNA: DA vs. Traditional LLNA	41	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
LLNA: DA vs. Human[4]	41	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
Traditional LLNA vs. Human[4]	41	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

[1] n = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.3 Accuracy Analysis (SI ≥ 3.0) Based on ICCVAM-recommended LLNA Performance Standards Reference Substances

In conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM), ICCVAM has developed internationally harmonized test method performance standards for the traditional LLNA (ICCVAM 2009),[11] which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Since the validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards, the LLNA: DA is not being evaluated using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the LLNA: DA test substances to the ICCVAM-recommended LLNA performance standards test substances are shown to provide a general comparison to a set list of reference substances (18 required reference substances and four optional reference substances) that represent a diverse substance group.

As shown in **Table C-4**, all of the 18 required reference substances and three of the four optional reference substances included in the ICCVAM-recommended LLNA performance standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) predicted the same sensitization classification for 16 of the 18 required ICCVAM-recommended reference substances tested. One discordant substance, 2-mercaptobenzothiazole, was classified as a sensitizer based on traditional LLNA results (EC3 = 1.7%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, *N,N*-dimethylformamide (DMF) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for 2-mercaptobenzothiazole. The positive result for 2-mercaptobenzothiazole reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 1%, 3%, and 10% (Gerberick et al. 2005). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 10%, 25%, and 50% (Idehara et al. 2008). The highest dose tested for 2-mercaptobenzothiazole in the traditional LLNA was the lowest dose tested in the LLNA: DA (10%) and resulted in an SI of 8.6 versus 2.0, respectively.

Notably, a review of the original LLNA: DA laboratory records for 2-mercaptobenzothiazole indicated that the concurrent positive control (10% eugenol in DMF) failed to yield an SI ≥ 3.0. Consequently the test method developers should have repeated the test for 2-mercaptobenzothiazole to ensure that the result obtained was correctly classified as negative and not the result of a failed experiment. This could explain the discordant result obtained between the traditional LLNA and the LLNA: DA test method for this test substance.

The second discordant substance, methyl methacrylate, was classified as a sensitizer based on traditional LLNA results (EC3 = 90%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, acetone: olive oil (AOO; 4:1) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for methyl methacrylate. The positive result for methyl methacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 10%, 30%, 50%, and 100% (Betts et al. 2006). By comparison, the negative result for methyl methacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 25%, 50%, 75%, and 100% (Idehara unpublished). The highest dose tested for methyl methacrylate in the traditional LLNA was the same in the LLNA: DA (100%) and resulted in an SI of 3.6 versus 1.8, respectively.

[11] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htmH.

As shown in **Table C-4**, when compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 predicted the same sensitization for all three of the optional reference substances tested. The optional reference substances, SLS and ethylene glycol dimethacrylate, were categorized as nonsensitizers based on GP and human data but as sensitizers by the LLNA: DA. Thus, similar to the traditional LLNA, these substances were false positive in the LLNA: DA. SLS was tested in the same vehicle (DMF) in both the traditional LLNA and the LLNA: DA. In addition, the positive results for SLS reported in the ICCVAM-recommended LLNA performance standards were based on five LLNA studies that tested SLS at 1%, 2.5%, 5%, 10%, and 20% (Loveless et al. 1996). In comparison, the positive result for SLS obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 1%, 2.5%, 5%, and 10% (Idehara et al. 2008). The EC3 values for SLS in the traditional LLNA (8.1%) and the LLNA: DA (6.9%) were comparable. In addition, ethylene glycol dimethacrylate was tested in the same vehicle (methyl ethyl ketone) in both the traditional LLNA and the LLNA: DA. The positive result for ethylene glycol dimethacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA study that tested the substance at 10%, 25%, and 50% (Gerberick et al. 2005). In comparison, the positive result for ethylene glycol dimethacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that also tested the substance at 10%, 25%, and 50% (Idehara unpublished). The EC3 values for ethylene glycol dimethacrylate in the traditional LLNA (28%) and the LLNA: DA (34%) were comparable.

Lastly, the optional reference substance, nickel (II) chloride, was categorized as a sensitizer based on GP and human data but as a nonsensitizer by the LLNA: DA. Thus, similar to the traditional LLNA, this substance was false negative in the LLNA: DA. Nickel (II) chloride was tested in the same vehicle (dimethyl sulfoxide [DMSO]) in both the traditional LLNA and the LLNA: DA. In addition, the negative results for nickel (II) chloride reported in the ICCVAM-recommended LLNA performance standards were based on two independent LLNA studies that tested the substance at 0.5%, 1%, and 2.5% (Basketter et al. 1999a) and at 1%, 2.5%, and 5% (Basketter and Scholes 1992). In comparison, the negative result for nickel (II) chloride obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 2.5%, 5%, and 10% (Idehara unpublished). The highest dose tested for nickel (II) chloride in the traditional LLNA was the same in the LLNA: DA (5%) and resulted in an SI of 2.4 versus 1.3, respectively.

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009 (27.7)	1	DMF	+	0.03 (7.5)	1
2,4-Dinitrochlorobenzene	AOO	+	0.049 (43.9)	15	AOO	+	0.08 (15.1)	11
4-Phenylenediamine	AOO	+	0.110 (26.4)	6	AOO	+	0.07 (5.1)	1
Cobalt chloride	DMSO	+	0.600 (7.2)	2	DMSO	+	1.27 (20.6)	5

continued

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value) (continued)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol	AOO	+	1.540 (31.0)	47	AOO	+	2.94 (12.4)	4
2-Mercaptobenzothiazole	***DMF***	**+**	***1.700 (8.6)***	***1***	***DMF***	**-**	***NA (2.0)***	***1***
Citral	AOO	+	9.170 (20.5)	6	AOO	+	15.63 (4.4)	1
Hexyl cinnamic aldehyde	AOO	+	9.740 (20.0)	21	AOO	+	11.10 (10.2)	18
Eugenol	AOO	+	10.090 (17.0)	11	AOO	+	4.50 (7.1)	1
Phenyl benzoate	AOO	+	13.600 (11.1)	3	AOO	+	2.26 (4.2)	1
Cinnamic alcohol	AOO	+	21.000 (5.7)	1	AOO	+	21.34 (5.7)	1
Imidazolidinyl urea	DMF	+	24.000 (5.5)	1	DMF	+	18.77 (4.7)	1
Methyl methacrylate	***AOO***	**+**	***90.000 (3.6)***	***1***	***AOO***	**-**	***NA (1.8)***	***1***
Chlorobenzene	AOO	-	NA (1.7)	1	AOO	-	NA (2.4)	1
Isopropanol	AOO	-	NA (1.7)	1	AOO	-	NA (2.0)	11
Lactic acid	DMSO	-	NA (2.2)	1	DMSO	-	NA (1.1)	5
Methyl salicylate	AOO	-	NA (2.9)	9	AOO	-	NA (1.8)	4
Salicylic acid	AOO	-	NA (2.5)	1	AOO	-	NA (2.0)	1
Sodium lauryl sulfate	DMF	FP	8.1 (8.9)	5	DMF	+	6.88 (3.4)	1
Ethylene glycol dimethylacrylate	MEK	FP	28.000 (7.0)	1	MEK	+	34.03 (4.5)	1
Xylene	AOO	FP	95.800 (3.1)	1	NT	NT	NT	NT
Nickel (II) chloride	DMSO	FN	NA (2.4)	2	DMSO	-	NA (1.3)	1

Bolded and italicized text highlights discordant LLNA: DA vs. traditional LLNA test results.

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; FN = false negative in traditional LLNA when compared to guinea pig and/or human results; FP = false positive in traditional LLNA when compared to guinea pig and/or human results; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; MEK = methyl ethyl ketone; NA = not applicable (stimulation index < 3.0); NT = not tested; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). The table lists the 18 required reference substances first (sorted from lowest to highest EC3 value), followed by the four optional reference substances (sorted from lowest to highest EC3 value).

[2] Substances tested in LLNA: DA intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or two-phased interlaboratory validation study (Omori et al. 2008).

[3] Based on mean EC3 value when more than one value was available. Numbers in parentheses indicate the maximum SI.

[4] Number of LLNA studies from which data were obtained.

Table C-5 provides the range and characteristics for 44 substances tested in the LLNA: DA based on sufficient traditional LLNA data. These substances are compared to the range of 18 required reference substances included on the ICCVAM-recommended LLNA performance standards reference substances list (ICCVAM 2009). The table indicates that the range of the substances tested in the LLNA: DA is similar to that included in the performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: DA in each of the categories included in the table.

Table C-5 Characteristics of the Substances Tested in the LLNA: DA Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1]

EC3 Range in the Traditional LLNA (%)	No. Substances	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/Min/Low/Unk)[3]
<0.1	**5**	**3/3[4]**	**0.009-0.083**	**5**	**4/0/0/0/1**
	2	1/1	0.009-0.049	2	2/0/0/0/0
≥0.1 to <1	**6**	**5/1**	**0.110-0.600**	**6**	**1/2/0/0/3**
	2	2/0	0.110-0.600	2	0/0/0/0/2
≥1 to <10	**11**	**6/5**	**1.540-9.740**	**10**	**4/0/3/1/3**
	4	1/3	1.540-9.740	4	2/0/1/0/1
≥10 to <100	**10**	**4/6**	**10.090-90.000**	**10**	**2/1/0/1/6**
	5	3/2	10.090-90.000	5	0/1/0/0/4
Negative	**12**	**7/5**	**NA**	**10**	**0/0/8/1/3**
	5	1/4	NA	3	0/0/2/0/3
Overall	**44**	**25/20[4]**	**0.009-90.000**	**41**	**11/3/11/3/16**
	18	8/10	0.009-90.000	16	4/1/3/0/10

Boldface represents characteristics of the LLNA: DA database, which includes the 44 substances with adequate traditional LLNA data, tested in the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or the two-phased interlaboratory validation study (Omori et al. 2008).

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; NA = not applicable because maximum stimulation index < 3.0; No. = number; Min = minimal; Mod = moderate; Unk = unknown.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm), based on the 18 required reference substances.

[2] Based on traditional LLNA studies for substances tested in the LLNA: DA (bold values) and for the 18 required reference substances in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

[3] Data obtained from Gerberick et al. (2007).

[4] One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

6.4.1 Discordance Between the LLNA: DA and the Traditional LLNA

When the outcomes for the 44 substances tested in the LLNA: DA (using SI ≥ 3.0) and the traditional LLNA were compared, the classifications for four substances were different. The LLNA: DA classified 3-aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate as nonsensitizers while the traditional LLNA classified them as sensitizers (**Tables C-6** and **C-7**). These substances were tested in the same vehicle in both the LLNA: DA and the traditional LLNA tests. One commonality noted between three of the four discordant substances is that they are solids. Furthermore, the molecular weights for 3-aminophenol and methyl methacrylate are both about 100 g/mol and those for 2-mercaptobenzothiazole and nickel (II) sulfate hexahydrate are comparable at 160 g/mol (**Annex II**). In addition, all four discordant substances are considered nonirritants based on GP data (**Table C-6**).

6.4.2 Discordance Among the LLNA: DA, the Traditional LLNA, and/or the Guinea Pig Test

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data, the LLNA: DA at SI ≥ 3.0 classified three substances differently compared with the traditional LLNA (**Table C-6**). 2-Mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and GP tests classified these substances as sensitizers. The discordant substances were tested at the same or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-6**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- 2-Mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- 2-Mercaptobenzothiazole and nickel (II) sulfate hexahydrate exist as solids and methyl methacrylate exists as a liquid.
- Nickel (II) sulfate hexahydrate and methyl methacrylate are soluble in water whereas 2-mercaptobenzothizole is not.

- All three discordant substances have similar molecular weights (approximately 100 to 160 g/mol).
- 2-Mercaptobenzothaizole has high peptide reactivity, whereas the peptide reactivity for methyl methacrylate and nickel (II) sulfate hexahydrate is not known.
- All three discordant substances are classified as sensitizers by the traditional LLNA (EC3 values were 90% for methyl methacrylate, 1.7% for 2-mercaptobenzothiazole, and 4.8% for nickel [II] sulfate hexahydrate).
- All three discordant substances are nonirritants based on data from GP studies (**Table C-6**).

In addition, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and SLS were positive in both the LLNA: DA and the traditional LLNA, but were negative in GP tests (**Table C-6**). In contrast, nickel (II) chloride was negative in both the LLNA: DA and the traditional LLNA but was positive in GP tests. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), and potential for skin irritation (**Annex III**) as follows:

- Benzalkonium chloride is an amine, ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Resorcinol and SLS exist as solids in their physical state and ethyl acrylate and ethylene glycol dimethacrylate exist as liquids in their physical state, whereas benzalkonium chloride can exist in both a solid and liquid physical state; nickel (II) chloride exists as a solid in its physical state.
- These five substances have varying molecular weights (100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, 198 g/mol for ethylene glycol dimethacrylate, and 288 g/mol for SLS); the molecular weight for nickel (II) chloride is about 130 g/mol.
- These five discordant substances are soluble in water; nickel (II) chloride is slightly soluble in water.
- Peptide reactivity is identified as minimal for resorcinol, and high for ethyl acrylate and ethylene glycol dimethacrylate, but is not identified for benzalkonium chloride and SLS; peptide reactivity for nickel (II) chloride is also not identified.
- Benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans, while resorcinol is considered a nonirritant based on studies in humans, and ethyl acrylate and ethylene glycol dimethacrylate are considered nonirritants based on studies in GPs; nickel (II) chloride is identified as negative at ≤0.15% based on GP studies (**Table C-6**).

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Benzalkonium chloride (0.07%)	AOO ACE[6]	+ (6.7, 2.5%)	+ (11.1, 2%)[7]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.2, 50%)[8]	+ (4.0, 50%)	-	Nonirritant at 0.3 Molar (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)

continued

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 Molar (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration test, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Tested in AOO in LLNA: DA and ACE in traditional LLNA.

[7] Highest SI occurred at concentration 1%.

[8] Highest SI occurred at concentration 25%.

[9] Highest SI occurred at concentration 10%.

6.4.3 Discordance Among the LLNA: DA, Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes, the LLNA: DA classified four substances differently compared with the classification of the traditional LLNA (**Table C-7**). 3-Aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and human outcomes classified these substances as

sensitizers. All four discordant substances were tested at similar or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- 3-Aminophenol is an amine and phenol compound, 2-mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is a carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- All four discordant substances exist as solids in their physical state except methyl methacrylate, which is a liquid.
- All four discordant substances are soluble in water except 2-mercaptobenzothizole.
- Molecular weights range from 100 to 167 g/mol.
- 2-Mercaptobenzothaizole has high peptide reactivity and 3-aminophenol has minimal peptide reactivity; peptide reactivity information for methyl methacrylate and nickel (II) sulfate hexahydrate is not available.
- All four discordant substances are classified as sensitizers by the traditional LLNA (EC3 values are 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate).
- All four discordant substances are classified as nonirritants based on data from GP studies, although human data indicate that nickel (II) sulfate hexahydrate is an irritant at 10% (**Table C-7**).

In addition, the LLNA: DA predicted the same outcome for SLS as the traditional LLNA (i.e., sensitizer), but was discordant when compared to the negative human test result (**Table C-7**). Diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben and sulfanilamide were also predicted similarly by the LLNA: DA and the traditional LLNA (i.e., nonsensitizers) but were discordant when compared to the positive human test result (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is a cyclic hydrocarbon and sulfur compound.
- SLS exists as a solid in its physical state; diethyl phthalate and isopropanol are liquids in their physical state, whereas nickel (II) chloride, propylparaben, and sulfanilamide exist as solids in their physical state.
- These substances have varying molecular weights that range from 60 to 222 g/mol for diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide to 288 g/mol for SLS.
- SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water and propylparaben is not.
- Diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity data for nickel (II) chloride and SLS are not available.
- SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered negative or nonirritants based on studies in rabbits or GP (**Table C-7**).

Table C-7 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Diethyl phthalate	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[6]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+ (HMT, data expressed as nickel)	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[7]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[8]	+ (20/25 at 25%)	Nonirritant at 25% (humans)
3-Aminophenol (3.2%)	AOO	- (2.8, 10%)	+ (5.7, 10%)	+	Nonirritant at 5% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+ (24/63 at 25%)	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+ (23/88 at 1%)	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 5%.

[8] Highest SI occurred at concentration 10% and 25%.

[9] Highest SI occurred at concentration 10%.

6.5 Accuracy Analysis Using Single Alternative Decision Criteria

In addition to the accuracy analysis using SI ≥ 3.0 to classify substances as sensitizers, other decision criteria were evaluated on the LLNA: DA test method performance, using the traditional LLNA (SI ≥ 3.0) as the comparative test (**Annex III**). The performance characteristics presented in this section are for 14 decision criteria that were used to determine whether the skin sensitization potential for the substances were positive (i.e., sensitizing) or negative (i.e., nonsensitizing). The substances evaluated were the 44 substances discussed in **Section 6.1** with both LLNA: DA and adequate comparative traditional LLNA data. The decision criteria analyzed included the following:

1. SI values ≥1.3, ≥1.5, ≥1.8, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0
2. Log-transformed ATP values of treated groups statistically different from control group based on analysis of variance (ANOVA) with a post-hoc Dunnett's test, when multiple treatment groups were tested, or Student's *t*-test when there was only one dosed group
3. Mean ATP values of treated groups ≥95% confidence interval (CI) of the control group mean
4. Mean ATP values of treated groups ≥2 standard deviations (SD) or ≥3 SD from the control group mean

Multiple tests were available for 14 substances tested with the LLNA: DA. The results for each of these substances were combined so that each substance was represented by one positive or negative result for each criterion evaluated for the accuracy analyses. The results were combined in three ways and a separate accuracy analysis was performed for each approach.

1. The positive/negative outcome for each substance was the most prevalent outcome for each criterion. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
3. The positive/negative outcome for each substance was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

The analysis using the most prevalent outcome for substances with multiple tests is presented in this section; the analyses using the highest maximum SI and the lowest maximum SI are included in **Annex V**.

When combining multiple test results for a single substance based on the most prevalent outcome, using the decision criterion of SI ≥ 3.0 to identify sensitizers, the 44 substances analyzed yielded an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-8)**. The decision criterion of SI ≥ 2.5 was similar to SI ≥ 3.0 in its performance characteristics. In comparison, the decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for specificity, which remained at 100% (12/12), and the false positive rate, which remained at 0% (0/12) (**Figure C-1** and **Table C-8**). Specifically, at SI ≥ 5.0, accuracy decreased to 57% (25/44) and the false negative rate increased to 59% (19/32).

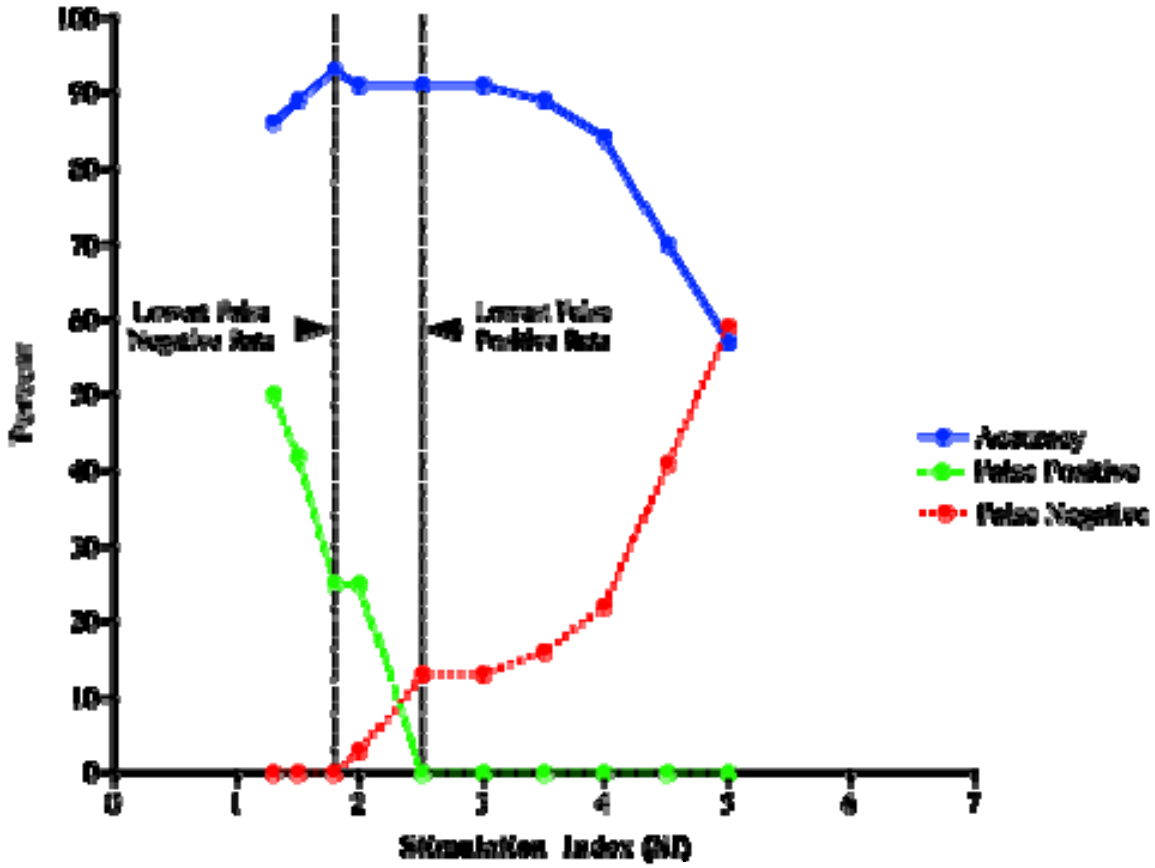
The decision criteria using lower SI values, SI ≥ 1.5 and SI ≥ 1.3, also decreased performance compared to SI ≥ 3.0 except for sensitivity, which increased to 100% (32/32), and the false negative rate, which decreased to 0% (0/32) (**Figure C-1** and **Table C-8**). Further, compared to SI ≥ 3.0, the lower SI cutoff of 2.0 had the same accuracy (91% [40/44]) but had an increased sensitivity of 97% (31/32), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 3% (1/32) (**Figure C-1** and **Table C-8**). Notably, the SI decision criterion that exhibited optimum performance characteristics compared to SI ≥ 3.0 was SI ≥ 1.8 (**Figure C-1** and **Table C-8**). Compared to SI ≥ 3.0, the lower SI cutoff of 1.8 had increased accuracy (93% [41/44]) and sensitivity (100% [32/32]), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 0% (0/32) (**Figure C-1** and **Table C-8**).

Use of ANOVA and summary statistics (i.e., mean ATP values of treated groups ≥95% confidence interval of the control group mean, or ≥2 or 3 SD from the control group mean), yielded accuracy values of 75 to 84%, with sensitivity values of 88 to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8 to 58% and the false positive rates were 42 to 92%. None of the statistical criterion evaluated exhibited increased performance characteristics when compared to SI ≥ 3.0 (**Table C-8**).

An evaluation to determine the robustness of the optimum SI ≥ 1.8 criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results (see **Annex VI**). Since the decision criterion of SI ≥ 1.8 showed optimum performance (i.e., increased accuracy and sensitivity, and decreased false negative rate compared to SI ≥ 3.0), it was further compared to SI ≥ 3.0 for accuracy against GP and human data (**Table C-9)**. When the LLNA: DA was compared to GP outcomes for substances with LLNA: DA, traditional LLNA, and GP data (40 substances), SI ≥ 1.8 had increased accuracy (80% [32/40] vs. 78% [31/40]), increased sensitivity (96% [25/26] vs. 85% [22/26]) and decreased specificity (50% [7/14] vs. 64% [9/14]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [7/14] vs. 36% [5/14]) and the false negative rate was decreased (4% [1/26] vs. 15% [4/26]) for SI ≥ 1.8 compared to SI ≥ 3.0. The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict GP outcomes was less (see **Table C-9**).

When the LLNA: DA was compared to human outcomes for substances with LLNA: DA, traditional LLNA, and human data (41 substances), SI ≥ 1.8 increased the accuracy (80% [33/41] vs. 76% [31/41]) and sensitivity (86% [30/35] vs. 74% [26/35]) and decreased the specificity (50% [3/6] vs. 83% [5/6]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [3/6] vs. 17% [1/6]) and the false negative rate was decreased (14% [5/35] vs. 26% [9/35]). The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict human outcomes was less (see **Table C-9**).

Figure C-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative SI Based on the Most Prevalent Outcome for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results in the LLNA: DA, the results for each substance were combined by using the most prevalent outcome. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

Table C-8 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bold text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table C-9 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Comparing Decision Criteria of SI ≥ 3.0 versus SI ≥ 1.8 Based on the Most Prevalent Outcome for Substances with Multiple Tests

Comparison	n[1]	Decision Criterion	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	SI ≥ 3.0	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
		SI ≥ 1.8	93 (41/44)	100 (32/32)	75 (9/12)	25 (3/12)	0 (0/32)	91 (32/35)	100 (9/9)
Substances with LLNA: DA, Traditional LLNA, and GP Data									
LLNA: DA vs. Traditional LLNA	40	SI ≥ 3.0	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
		SI ≥ 1.8	95 (38/40)	100 (30/30)	80 (8/10)	20 (2/10)	0 (0/30)	94 (30/32)	100 (8/8)
LLNA: DA vs. GP[3]	40	SI ≥ 3.0	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
		SI ≥ 1.8	80 (32/40)	96 (25/26)	50 (7/14)	50 (7/14)	4 (1/26)	78 (25/32)	88 (7/8)
Traditional LLNA vs. GP[3]	40	SI ≥ 3.0	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data									
LLNA: DA vs. Traditional LLNA	41	SI ≥ 3.0	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
		SI ≥ 1.8	95 (39/41)	100 (31/31)	80 (8/10)	20 (2/10)	0 (0/31)	94 (31/33)	100 (8/8)
LLNA: DA vs. Human[4]	41	SI ≥ 3.0	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
		SI ≥ 1.8	80 (33/41)	86 (30/35)	50 (3/6)	50 (3/6)	14 (5/35)	91 (30/33)	38 (3/8)
Traditional LLNA vs. Human[4]	41	SI ≥ 3.0	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

[1] n = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.6 Discordant Results for Accuracy Analysis Using Single Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-8** and **C-9,** in order to provide a comparison to the discordant substances identified when using the decision criterion of SI ≥ 3.0 to identify sensitizers. Discordant results for the alternative decision criteria are first discussed in general using the traditional LLNA as the reference test (**Section 6.6.1**) and then discordant results for SI ≥ 1.8, the single optimized alternative decision criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

6.6.1 Discordant Results Using Single Alternative Decision Criteria Compared with the Traditional LLNA

Table C-10 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the most prevalent outcome for the substances with multiple tests. Using SI ≥ 2.0 as the decision criterion resulted in three nonsensitizers in the traditional LLNA (chlorobenzene, hexane, and salicylic acid) being misclassified as sensitizers in the LLNA: DA. Also, methyl methacrylate, a sensitizer in the traditional LLNA, was misclassified as a nonsensitizer in the LLNA: DA. Using SI ≥ 1.8 as the decision criterion still resulted in chlorobenzene, hexane, and salicylic acid being misclassified as sensitizers in the LLNA: DA compared to the traditional LLNA, although methyl methacrylate was no longer misclassified as a nonsensitizer in the LLNA: DA compared to SI ≥ 2.0. As the SI decision criterion was further reduced to SI ≥ 1.5 and SI ≥ 1.3, two additional substances, 1-bromobutane and methyl salicylate, were also misclassified as sensitizers when compared to traditional LLNA results. In addition, using SI ≥ 1.3 also misclassified nickel (II) chloride as a sensitizer in the LLNA: DA compared to the traditional LLNA. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 5.0, 19 substances were discordant. As **Table C-10** shows, all 19 substances were sensitizers in the LLNA but misclassified as nonsensitizers in the LLNA: DA.

Use of a statistical test (i.e., ANOVA or *t*-test) to identify sensitizers misclassified two sensitizers in the traditional LLNA (2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers in the LLNA: DA and five nonsensitizers (1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers. Use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) generally misclassified nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Specifically, using ≥3 SD of vehicle control mean misclassified five nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben. Using treatment group absorbance ≥2 SD of vehicle control mean misclassified the same five substances as sensitizers, as well as methyl salicylate and salicylic acid. Using the treatment group absorbance ≥95% CI of vehicle control mean misclassified all the nonsensitizers misclassified as sensitizers in the LLNA: DA when using either ≥3 SD or ≥2 SD of vehicle control mean, as well as four additional substances: diethyl phthalate, dimethyl isophthalate, isopropanol, and lactic acid. In some instances, use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) misclassified sensitizers in the traditional LLNA as nonsensitizers in the LLNA: DA. Using ≥3 SD of vehicle control mean misclassified four traditional LLNA sensitizers as LLNA: DA nonsensitizers: butyl glycidyl ether, ethyl acrylate, methyl methacrylate, and propyl gallate. Using treatment group absorbance ≥2 SD of vehicle control mean only misclassified ethyl acrylate and propyl gallate as nonsensitizers in the LLNA: DA compared to the traditional LLNA and using the treatment group absorbance ≥95% CI did not misclassify any traditional LLNA sensitizers as LLNA: DA nonsensitizers.

6.6.2 Discordant Results for Accuracy Analysis Using a Single Optimized Alternative Decision Criterion (SI ≥ 1.8)

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data based on an SI ≥ 1.8, the LLNA: DA classified two substances (chlorobenzene and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-11**). Chlorobenzene and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and GP outcomes. In contrast, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and sodium lauryl sulfate were identified as sensitizers by the LLNA: DA similar to the traditional LLNA but as nonsensitizers based on GP outcomes. Further, nickel (II) chloride was identified as a nonsensitizer by the LLNA: DA similar to the traditional LLNA but as a sensitizer based on GP outcomes. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- Chlorobenzene is a halogenated hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; benzalkonium chloride is an amine (also an onium compound), ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Chlorobenzene exists as a liquid and salicylic acid exists as a solid in its physical state; benzalkonium chloride can exist in both a solid and liquid physical state, whereas ethyl acrylate and ethylene glycol dimethacrylate are liquids, and resorcinol and SLS are solids; nickel (II) chloride is a solid.
- Chlorobenzene has a molecular weight of 113 g/mol and salicylic acid has a molecular weight of 138 g/mol; the five substances that are concordant with the traditional LLNA but discordant with GP outcomes have varying molecular weights that range from 100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, and 198 g/mol for ethylene glycol dimethacrylate to 288 g/mol for SLS; the molecular weight for nickel (II) chloride is 130 g/mol.
- All the discordant substances are soluble in water.
- Chlorobenzene has minimal peptide reactivity while peptide reactivity data for salicylic acid are not available; the peptide reactivity for resorcinol is identified as minimal, and that for ethyl acrylate and ethylene glycol dimethacrylate is high while peptide reactivity data for benzalkonium chloride and SLS are not available; peptide reactivity data for nickel (II) chloride are not available.
- Benzalkonium chloride (EC3 = 0.07%), ethyl acrylate (EC3 = 32.8%), ethylene glycol dimethacrylate (EC3 = 28%), resorcinol (EC3 = 6.33%), and SLS (EC3 = 8.08%) are identified as sensitizers by the traditional LLNA.
- Chlorobenzene has low irritancy potential assumed based on clinical literature while salicylic acid is an irritant at 20% in mice; benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans and ethyl acrylate, ethylene glycol dimethacrylate, and resorcinol are considered nonirritants based on studies in humans or GP; nickel (II) chloride is considered a negative at ≤0.15% based on GP data (**Table C-11**).

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes based on an SI ≥ 1.8, the LLNA: DA classified two substances (hexane and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-12**). Hexane and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and human outcomes. Further, SLS was classified as a sensitizer by the LLNA: DA and traditional LLNA but as a nonsensitizer based on human outcomes. In contrast,

diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide were all classified as nonsensitizers by the LLNA: DA and the traditional LLNA but as sensitizers based on human outcomes (**Table C-12**). In instances where the substances were discordant in the LLNA: DA compared to the traditional LLNA, the discordant substances were tested at the same maximum concentration. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- Hexane is an acyclic hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is sulfur compound.
- Hexane is a liquid and salicylic acid is a solid; SLS is a solid; diethyl phthalate and isopropanol are liquids while nickel (II) chloride, propylparaben, and sulfanilamide are solids.
- Hexane and salicylic acid have molecular weights of 86 g/mol and 138 g/mol, respectively; the molecular weight for SLS is 288 g/mol; the other discordant substances have varying molecular weights that range from 60 g/mol for isopropanol, 130 g/mol for nickel (II) chloride, 172 g/mol for sulfanilamide, and 180 g/mol for propylparaben to 222 g/mol for diethyl phthalate.
- Hexane, salicylic acid, SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water; propylparaben is not.
- Hexane, diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity information for salicylic acid, nickel (II) chloride, and SLS is not available.
- SLS is identified as a sensitizer by the traditional LLNA (EC3 = 8.08%).
- Hexane has been found to be an irritant at 100% in humans as has salicylic acid at 20% in mice; SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered to be nonirritants based on studies in rabbits, GP, or humans (**Table C-12**).

Table C-10 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Most Prevalent Outcome for Substances with Multiple Tests

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
3-Aminophenol (3.2%)					-	-	-	-	-	-				
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.91%)					-									
Citral (9.17%)					-	-								
Cobalt chloride (0.6%)					-	-								
Diethyl maleate (3.6%)					-	-	-							
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)		+												
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28%)					-	-								
Formaldehyde (0.5%)					-									
Hexane (-)	+	+	+	+							+	+	+	+
Imidazolidinyl urea (24%)					-									
Isopropanol (-)		+												
Lactic acid (-)		+												
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Methyl methacrylate (90%)	-		-	-	-	-	-	-	-	-	-			
Methyl salicylate (-)		+	+										+	+
Nickel (II) chloride (-)		+	+	+										+
Nickel (II) sulfate hexahydrate (4.8%)					-	-	-	-	-	-				
Phenyl benzoate (13.6%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.33%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sulfanilamide (-)	+													
Sodium lauryl sulfate (8.08%)					-	-	-	-						
Trimellitic anhydride (4.71%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 value for sensitizers.

[2] LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[3] Analysis of variance assessed differences of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. Significance by analysis of variance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table C-11 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and GP Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Chlorobenzene (-)	AOO	+ (2.4, 25%)	- (1.7, 10%)[6]	-	No data. Low irritancy potential assumed based on clinical literature.
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Benzalkonium chloride (0.07%)	AOO ACE[7]	+ (6.7, 2.5%)	+ (11.1, 2%)[8]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.3, 50%)[6]	+ (4.0, 50%)	-	Nonirritant at 0.3 M (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous ; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Highest SI occurred at concentration 25%.

[7] Benzalkonium chloride tested in AOO vehicle in LLNA: DA and ACE vehicle in traditional LLNA.

[8] Highest SI occurred at concentration 1%.

[9] Highest SI occurred at concentration 10%.

Table C-12 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Hexane (-)	AOO	+ (2.3, 100%)	- (2.2, 100%)	- (0/25 at 100%)	Irritant at 100% (humans)
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Diethyl phthalate (-)	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol (-)	AOO	- (1.97, 50%)	- (1.7, 50%)[7]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
Propylparaben (-)	AOO	- (1.3, 25%)	- (1.4, 25%)[8]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide (-)	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[9]	+	Nonirritant at 25% (humans)

Abbreviations: aq. = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 10%.

[8] Highest SI occurred at concentration 5%.

[9] Highest SI occurred both at concentration 10% and at concentration 25%.

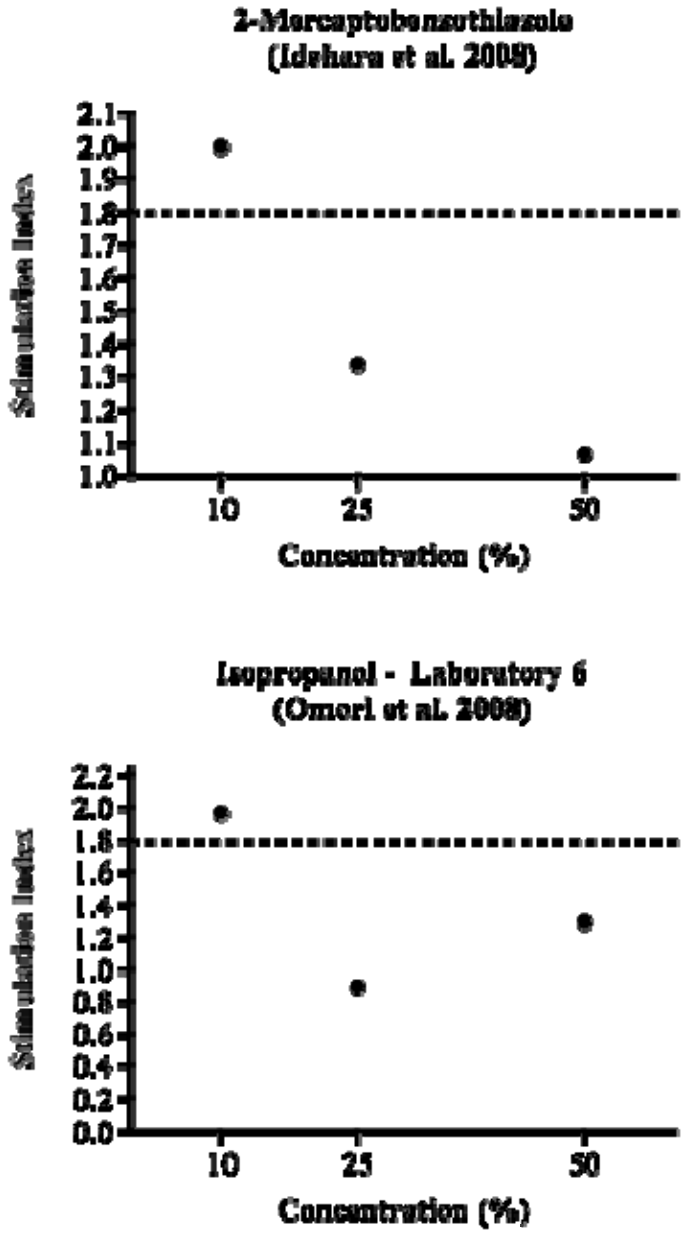
6.7 Accuracy Analysis for the Reduced LLNA: DA Using the SI ≥ 1.8 Decision Criterion

An accuracy analysis for the rLLNA: DA was performed using the optimized SI ≥ 1.8 criterion to identify sensitizers. The rLLNA: DA uses only the highest dose of the test substance that does not produce excessive skin irritation and/or systemic toxicity; the two lower dose groups are not used. The available validation database for the rLLNA: DA analysis included 123 individual tests that used multiple doses. The performance of the rLLNA: DA was evaluated by comparing the outcome of the highest dose for each test to the outcome of the same test when considering all doses tested. Using SI ≥ 1.8 to identify sensitizers, the accuracy of the rLLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the multiple-dose LLNA: DA. One study that tested 2-mercaptobenzothiazole at 10%, 25%, and 50% produced a maximum SI value of 2.00 at the lowest dose tested (**Figure C-2**). The second false negative test was for isopropanol at 10%, 25%, and 50%, which produced the maximum SI of 1.97 at the lowest dose tested (**Figure C-2**).

6.8 Analyses Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5,** the accuracy of the LLNA: DA when using various single alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA (SI ≥ 3.0), the optimum performance (i.e., accuracy of 93% [41/44] and sensitivity of 100% [32/32]) was achieved using the decision criterion of SI ≥ 1.8 (**Table C-8**). Although the SI ≥ 1.8 produced a false positive rate of 25% (3/12) it yielded a false negative rate of 0% (0/32) (**Table C-8**). Increasing the SI decision criterion to SI ≥ 2.5 decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The 0% false positive rate using SI ≥ 2.5 and the 0% false negative rate using SI ≥ 1.8 prompted an evaluation using two SI decision criteria for determining LLNA: DA results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.8). This evaluation is described in detail in **Annex VII**.

Figure C-2 Dose Response Curves for Tests Identified as Sensitizers by the LLNA: DA but as Nonsensitizers by the Reduced LLNA: DA



Note: The horizontal line in each figure indicates an SI ≥ 1.8, which is the threshold that is considered optimum for providing a positive response in the LLNA: DA. Points on or above this line would indicate a positive (sensitizer) response, while points below this line would indicate a negative (nonsensitizer) response.

7.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances, and indicates the extent to which a test method can be transferred successfully among laboratories. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.5** showed that SI ≥ 1.8 was the SI value that produced the most optimum results (i.e., accuracy of 93% [41/44], sensitivity of 100% [32/32], and false negative rate of 0% [0/32]) among the alternative decision criteria evaluated when the traditional LLNA was the reference test (**Table C-8)**. Thus, this section provides an assessment of reproducibility for the decision criterion of SI ≥ 1.8 to identify sensitizers. For additional reproducibility analyses using a single decision criterion see **Annex VIII,** which describes the evaluation of reproducibility for the decision criterion of SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) and SI ≥ 2.0 (previously evaluated as an optimum decision criterion in the March 2009 revised draft BRD evaluated by the Panel) to identify sensitizers. Further, the reproducibility analyses based on the evaluation of multiple decision criteria briefly mentioned in **Section 6.8** (i.e., SI ≥ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI ≤ 1.8 to identify nonsensitizers) is detailed in **Annex VII**.

7.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-13**). The data indicate CV values of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). NICEATM also determined the intralaboratory reproducibility of EC1.8 values (estimated concentration needed to produce an SI of 1.8) for the same set of data. This resulted in CV values of 36% and 23% for isoeugenol and eugenol indicating larger intralaboratory variability compared to EC3 values with CV values of 21% and 11% for isoeugenol and eugenol, respectively.

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98

continued

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1] (continued)

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	**3.40%**	**2.35%**	**2.46%**
EC1.8	**0.69%**	**1.23%**	**0.69%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC1.8: 0.87% ± 0.31% and 36% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**
EC1.8	**4.20%**	**3.30%**	**2.63%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC1.8: 3.38% ± 0.79% and 23% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

7.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Annex IV**) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-14)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle since two metals dissolved in DMSO (cobalt chloride and nickel (II) sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories

(Table C-15). One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC1.8 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.2.1**) and a CV analysis for the quantitative results (EC1.8 values) (**Sections 7.2** and **7.3**).

Table C-14 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table C-15 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-16** for SI ≥ 1.8. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), nine substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were three substances with discordant results between the labs (isopropanol, 3-aminophenol and nickel [II] sulfate hexahydrate). The interlaboratory concordance for isopropanol was 90% (9/10) and the one discordant lab reported a maximum SI = 1.97 at the lowest dose tested. The interlaboratory concordance for 3-aminophenol and nickel (II) sulfate hexahydrate was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 1.8 at the middle dose tested (SI = 2.32 and SI = 1.99 at 10%) and one laboratory did not achieve SI ≥ 1.8 at any dose tested (**Annex IV**). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 1.8 at all three doses tested (**Annex IV**). Notably, when analyzing the dose response curves for the three tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-16 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitro-chlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	**- (1.54)**	**- (0.91)**	**- (1.01)**	**- (1.57)**	**- (0.76)**	**+ (1.97)**	**- (1.45)**	**- (1.21)**	**- (0.70)**	**- (1.25)**	**9/10**
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-17**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 1.8 at any dose, while one laboratory produced an SI ≥ 1.8 at the lowest dose tested. Nickel (II) sulfate hexahydrate was also tested in the first phase of the interlaboratory validation study where interlaboratory concordance was 67% (2/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-17 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Bolded substance did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses,

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.2 Interlaboratory Reproducibility – EC1.8 Values

The quantitative (i.e., EC1.8 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results that yielded positive results (SI ≥ 1.8) during the first and second phases of the LLNA: DA interlaboratory validation study. The equation used for calculating EC1.8 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC1.8 = c + \left[\frac{(1.8 - d)}{(b - d)}\right] \times (a - c)$$

where the data points lying immediately above and below the SI = 1.8 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 1.8, an EC1.8 value was extrapolated according to the equation:

$$EC1.8_{ex} = 2^{\left[\log_2 a - \frac{b}{(b-d)} \times (\log_2 a - \log_2 c)\right]}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC1.8 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phases of the interlaboratory validation study are shown in **Tables C-19** and **C-20**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71% (**Table C-18**). In the second phase of the interlaboratory validation study, CV values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49% (**Table C-19**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentrations needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, eight laboratories reported EC1.8 values outside the acceptance range indicated for 2,4-dinitrochlorobenzene; all of the eight laboratories obtained EC1.8 values that were lower than the specified acceptance range (<0.025%) (**Table C-18**). For hexyl cinnamic aldehyde, all the laboratories participating in the first phase of the interlaboratory validation study obtained an EC1.8 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and five of the seven laboratories obtained EC1.8 values that were within the acceptance range indicated (**Table C-19**).

Table C-18 EC1.8 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name	EC1.8 (%)										Mean EC1.8 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.018 (11.97)**	**0.018 (9.23)**	**0.023 (9.96)**	**0.014 (8.53)**	**0.081 (7.86)**	**0.014 (15.14)**	**0.006 (13.18)**	**0.017 (12.60)**	**0.012 (10.89)**	**0.077 (4.71)**	**0.028 ± 0.027**	**97**
Hexyl cinnamic aldehyde (+)	**6.358 (5.78)**	**6.687 (4.82)**	**7.346 (4.44)**	**5.884 (5.11)**	**9.597 (3.97)**	**5.961 (5.50)**	**5.479 (7.09)**	**5.783 (10.22)**	**8.457 (3.88)**	**6.508 (3.51)**	**6.806 ± 1.312**	**19**
Isopropanol (-)	NA	NA	NA	NA	NA	IDR	NA	NA	NA	NA	NA	NA
Abietic acid (+)		3.636				4.878	4.598				4.371 ± 0.651	15
3-Aminophenol (+)	1.175		NA					2.507			1.841 ± 0.942	51
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.337	4.082				0.265		1.561 ± 2.183	140
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.209	0.579			1.380						0.723 ± 0.599	83
Glutaraldehyde (+)	0.064	0.235			0.104						0.134 ± 0.089	67
Cobalt chloride[2] (+)				0.233[3]		0.025		0.071			0.110 ± 0.109	99
Nickel (II) sulfate hexahydrate (+)				NA		0.188		IDR			0.188 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.3% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC1.8 values that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5-20% for hexyl cinnamic aldehyde and 0.025-0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; IDR = insufficient dose response for calculation of EC1.8; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-19 EC1.8 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC1.8 (%)							Mean EC1.8 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**5.793 (4.47)**	**5.426 (5.71)**	**5.627 (5.41)**	**4.442 (7.60)**	**6.469 (3.92)**	**4.437 (8.42)**	**5.720 (6.45)**	**5.416 ± 0.741**	**14**
Cobalt chloride[2] (+)	3.499		1.382	0.723			0.393	1.499 ± 1.395	93
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		5.938		NA		5.938 ± NA	NA
Potassium dichromate (+)	0.089	0.089			0.046		0.041	0.066 ± 0.026	39

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC1.8 values (shaded cells) are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5-20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentrations needed to produce a stimulation index of 1.8; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC1.8 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8% to 83.7% for five substances tested in five laboratories (**Table C-20**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 97% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 19% and 14% for hexyl cinnamic aldehyde tested in the first and second phases of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 140% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-20 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
SLS	13.4	4.4	1.5	17.1	4.0	83.7

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.

[1] From ICCVAM 1999 report.

7.3 Reproducibility Analysis for Substances with Multiple Tests

Section 6.5 details the accuracy analysis for the LLNA: DA (using the most prevalent outcome for substances with multiple tests) when using one optimized criterion to classify substances as potential sensitizers (SI ≥ 1.8). SI ≥ 1.8 was evaluated for classifying substances as potential sensitizers because it resulted in no false negative results, with respect to traditional LLNA data. This section examines the reproducibility of the tests for the 14 substances that had multiple LLNA: DA test results, regardless of whether the tests were performed in one laboratory or multiple laboratories. The frequency with which SI values for the 14 substances occurred in one of three SI categories was considered. The three SI categories were:

- LLNA: DA nonsensitizers with SI < 1.8
- LLNA: DA sensitizers with SI between 1.8 and 2.5 (borderline positive results with potential to be false positives with respect to classification by the traditional LLNA)
- LLNA: DA sensitizers with SI ≥ 2.5

For the 14 substances, three to 18 tests were available. **Table C-21** shows the proportion of the tests for each substance that produced SI values in each category. For the four traditional LLNA nonsensitizers with multiple test results, there were 23 LLNA: DA tests that produced SI < 1.8 and one LLNA: DA test that produced an SI between 1.8 and 2.5. For the 10 traditional LLNA sensitizers with multiple LLNA: DA test results, however, SI values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were particularly variable: 50% (4/8) produced SI < 1.8 (four tests with SI = 0.79, 1.24, 1.52, and 1.56), 25% (2/8) produced 1.8 < SI < 2.5 (SI = 2.13 and 2.17), and 25% (2/8) produced SI ≥ 2.5 (SI = 3.49 and 11.78). 3-Aminophenol also produced SI values in all three categories: 33% (1/3) of the tests had SI < 1.8 (SI = 1.76), 33% (1/3) of the tests had 1.8 < SI < 2.5 (SI = 2.38), and 33% (1/3) of the tests had SI ≥ 2.5 (SI = 2.83). Cobalt chloride tests produced SI values in two categories: 12.5% (1/8) of the tests had 1.8 < SI < 2.5 (SI = 2.01) and seven of eight tests (87.5%) produced SI ≥ 2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the remaining seven traditional LLNA sensitizers were 100% concordant (**Table C-21**).

Table C-21 Concordance of LLNA: DA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	Total Tests
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional LLNA results.

8.0 LLNA: DA Data Quality

All of the studies included in this performance evaluation are based on individual animal data submitted to NICEATM in the form of original data and study records. Furthermore, manuscripts detailing the results for 31 substances evaluated in the intralaboratory study and 14 substances evaluated in the two-phased interlaboratory validation have been published in the peer-reviewed literature (Idehara et al. 2008; Omori et al. 2008). An independent audit has been conducted to confirm that the reported data from the intralaboratory validation study (assessment of 31 substances from Idehara et al. 2008) performed by Daicel Chemical Industries, Ltd. were the same as the data originally recorded (Idehara et al. 2008). The data from the two-phased interlaboratory validation study were not subjected to a formal audit, but the raw data were reportedly entered directly into formatted MS-Excel templates provided by the study management team prior to being used for analyses (Omori et al. 2007). Data recently received for 14 substances evaluated in an intralaboratory validation study (Idehara unpublished) were also not subjected to a formal audit. The intralaboratory assessment at Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), as well as the two-phased interlaboratory validation study (Omori et al. 2008), did not conduct their studies in compliance with Good Laboratory Practice guidelines, although all of the participating laboratories reportedly have this capability.

9.0 Other Scientific Reports and Reviews

Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative nonradioisotope LLNA test method. The manuscript details the determination of an optimal dosing schedule and further compares SI values obtained from lymph node weights versus ATP content to determine an appropriate lymphocyte proliferation endpoint. The authors further assess the intermediate precision and sensitivity/specificity of the LLNA: DA. In those experiments, four compounds (2,4-dinitrochlorobenzene, eugenol, α-hexyl cinnamic aldehyde, and methyl salicylate) were tested and no significant differences were noted in the SI levels generated from the LLNA: DA and the traditional LLNA. The studies by Yamashita et al. provided the basis for the expanded intralaboratory study of 31 substances performed by Daicel Chemical Industries, Ltd. and published by Idehara et al. (2008) (described in **Sections 6.0** and **7.0**).

Idehara et al. (2008) summarize the LLNA: DA test method in terms of test substance dosing schedule, preparation of single cell suspensions of the auricular lymph nodes, measurement of ATP content, and explanation of statistical analyses employed. The authors further describe how the results correlate between ATP content and lymph node cell number, the test results (i.e., mean SI values and EC3 values) obtained for the 31 substances, the concordance of the LLNA: DA versus the traditional LLNA EC3 values, and the reproducibility of EC3 and SI values. Based on the details included in the manuscript, the authors conclude that the SI values obtained from measuring ATP content were similar to the traditional LLNA and therefore the LLNA: DA was a promising nonradioisotope modified test method for evaluating the skin sensitization potential of substances.

Omori et al. (2008) describe the two-phased interlaboratory validation study used to evaluate the reliability and relevance of the LLNA: DA test method (see **Section 7.0**). They describe the organization and technology transfer of the test method between the laboratories, as well as test substance selection and allocation. They further describe the development of the LLNA: DA and the resulting standard protocol for the LLNA: DA interlaboratory study. They provide the interlaboratory data for analyzing both ATP content with regard to SI values and lymph node weight and discuss assay sensitivity and interlaboratory variability. Based on the data summarized in the manuscript, the authors conclude that in the first phase of the interlaboratory validation study, a large variation was observed for two substances (cobalt chloride and nickel [II] sulfate hexahydrate) but in the second phase of the interlaboratory validation study this variation was small. The authors attribute the initial variation to application of DMSO as the solvent for the metallic salts and therefore, prior to the second phase of the interlaboratory validation study, include operation of LLNA: DA with DMSO in the technology transfer seminar. In conclusion, the authors view the LLNA: DA as a reliable test method for predicting skin sensitization potential of substances.

Regarding the LLNA: DA test method, noncommission members of JaCVAM met on August 28, 2008 at the National Institute of Health Sciences, Tokyo, Japan, and endorsed the following statement: “Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA: DA coordinated by Japanese Society for Alternative to Animal Experiments, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the OECD testing guidelines No. 429 on skin sensitization: LLNA. The JaCVAM regulatory acceptance board has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by MHLW of the study prepared for the JaCVAM ad hoc peer review panel.”

10.0 Animal Welfare Considerations

The LLNA: DA will require the use of the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009). However, since the traditional LLNA uses radioactive materials and as such its use might be restricted in some countries and institutions due to the complications associated with storage, use, and disposal, broader use of a nonradioactive alternative to the traditional LLNA, such as the LLNA: DA, could further reduce the number of GPs that are used to assess skin sensitization.

Further, the LLNA: DA offers increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

10.1 Rationale for the Need to Use Animals

The rationale for the use of animals in the LLNA: DA is the same as the rationale for the traditional LLNA. There currently are no valid and accepted non-animal test methods to determine the ACD potential of substances and products, except for situations where human studies could be conducted ethically and where such studies would meet regulatory safety assessment requirements. Additionally, the most detailed information about the induction and regulation of immunological responses are available for mice (ICCVAM 1999).

10.2 Basis for Determining the Number of Animals Used

The number of animals used for the experimental, vehicle, and positive control groups is based on the number of animals used in the development (Yamashita et al. 2005) and validation of the test method (Idehara et al. 2008; Omori et al. 2008), which is the same as that specified in the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009).

10.3 Reduction Considerations

A further reduction of up to 40% (15 vs. 25) could be achieved by using a reduced version of the LLNA: DA, in cases where dose-response information is not needed for hazard identification purposes. In such an approach, only the highest dose of the test article that does not elicit excessive skin irritation or systemic toxicity would be administered, and the two lower dose groups would not be used. Additional reductions could be achieved by testing more substances concurrently, so that the same vehicle and positive control group could be used for multiple substances.

11.0 Practical Considerations

Several issues are taken into account when assessing the practicality of using an alternative to an existing test method. In addition to performance evaluations, assessments of the laboratory equipment and supplies needed to conduct the alternative test method, level of personnel training, labor costs, and the time required to complete the test method relative to the existing test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the existing test method it is intended to replace.

11.1 Transferability of the LLNA: DA

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure as well as laboratories with less or no experience in the particular procedure. It would be expected that the transferability of the LLNA: DA would be similar to the traditional LLNA, since their test method protocols are experimentally similar. Notably, the test method developer does indicate that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). The first and second phases of the interlaboratory validation study have demonstrated that this test method is transferable (see **Section 7.0**).

11.2 Laboratories and Major Fixed Equipment Required to Conduct the LLNA: DA

Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for handling radioactive materials. However, the LLNA: DA does require access to a luminometer capable of detecting light emission by ATP for the assessment of lymphocyte proliferation. The remaining requirements (e.g., animal care laboratories) are the same between the two methods.

11.3 LLNA: DA Training Considerations

The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA, although the LLNA: DA includes an additional requirement that users operate a luminometer instead of a scintillation counter and be able to process this data.

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13.0 Glossary

Accuracy:[12] (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *concordance* (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Allergic Contact Dermatitis (ACD): A Type IV allergic reaction of the skin that results from repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

Assay:[12] The experimental system used. Often used interchangeably with *test* and *test method*.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Concordance:[12] The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

EC1.8: The estimated concentration needed to produce a stimulation index of 1.8, as compared to the concurrent vehicle control.

EC3: The estimated concentration needed to produce a stimulation index of three, as compared to the concurrent vehicle control.

ECt: The estimated concentration needed to produce a stimulation index of a specific threshold, as compared to the concurrent vehicle control.

False negative:[12] A substance incorrectly identified as negative by a test method.

False negative rate:[12] The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive:[12] A substance incorrectly identified as positive by a test method.

False positive rate:[12] The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Good Laboratory Practices (GLP):[12] Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organisation for Economic Co-operation and Development (OECD) and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard[12]**:** The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility:[12] A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results.

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[12] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability:[12] The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility:[12] The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Immunological: Relating to the immune system and immune responses.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Lymphocyte: A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

Murine local lymph node assay (LLNA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure on the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of ^{3}H-thymidine or ^{125}I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure on the ear to the substance. The LLNA: DA is a nonradioactive modification of the traditional LLNA and assesses lymphocyte cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation.

Negative predictivity:[12] The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Nonsensitizer: A substance that does not cause skin sensitization following repeated skin contact.

Performance:[12] The accuracy and reliability characteristics of a test method (see *accuracy*, *reliability*).

Positive control: A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance are considered adequate by the OECD.

Positive predictivity:[12] The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence:[12] The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol:[12] The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria and procedures for the evaluation of the test data.

Quality assurance:[12] A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative:[12] A new or modified test method that reduces the number of animals required.

Reference test method:[12] The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative:[12] A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance:[12] The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability:[12] A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative:[12] A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility:[12] The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and interlaboratory reproducibility).

rLLNA: DA (reduced LLNA: DA): A variant of the LLNA: DA that employs a single, high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

Sensitivity:[12] The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Skin sensitizer: A substance that induces an allergic response following skin contact.

Specificity:[12] The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Stimulation index (SI): A value calculated for the LLNA: DA to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of the mean ATP content of the auricular lymph nodes from a group of treated mice to the mean ATP content of the auricular lymph nodes from a group of vehicle control mice. The mean ATP content is measured in relative luminescence units. For the LLNA: DA and the rLLNA: DA, an SI $\geq$ 1.8 classifies a substance as a potential skin sensitizer.

Test:[12] The experimental system used; used interchangeably with *test method* and *assay*.

Test method:[12] A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Transferability:[12] The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table:[12] The two-by-two table can be used for calculating accuracy (concordance) ([a + d]/[a + b+ c + d]), negative predictivity (d/[c + d]), positive predictivity (a/[a + b]), prevalence ([a + c]/[a + b + c + d]), sensitivity (a/[a + c]), specificity (d/[b + d]), false positive rate (b/[b + d]), and false negative rate (c/[a + c]).

		New Test Outcome		
		Positive	**Negative**	**Total**
Reference Test Outcome	**Positive**	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method:[12] An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation:[12] The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight-of-evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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